

IN THE CLAIMS

1. (currently amended) A method of producing a plurality of overlapping fragments of a size in the range of about 15-30 nucleotides, an hsiRNA mixture, comprising:

(a) digesting a preparation of large double-stranded RNA in a reaction mixture containing a divalent transition metal cation and a prokaryotic RNaseIII wherein the ratio of enzyme to substrate (w/w) is greater than or equal to about 0.25:1; and RNaseIII; and

(b) producing the plurality of overlapping fragments of a size in the range of about 15-30 nucleotides. hsiRNA mixture.

2. (currently amended) A method according to claim 1, wherein the plurality of overlapping fragments hsiRNA mixture is the product of complete digestion of the preparation of large double-stranded RNA.

3-4 (cancelled)

5. (original) A method according to claim 1, wherein the transition metal cation is manganese.

6. (original) A method according to claim 5, wherein the reaction mixture contains manganese ions at a concentration in the range of about 5-10 mM.

7. (original) A method according to claim 6, wherein the reaction mixture contains manganese ions at a concentration in the range of about 10-20 mM.
8. (original) A method according to claim ~~±3~~, wherein the transition metal is selected from nickel, cobalt and cadmium.
9. (original) A method according to claim 2, wherein the complete digestion is achieved in less than 6 hours.
10. (original) A method according to claim 2, wherein the complete digestion-is achieved in less than 2 hours.
11. (cancelled)
12. (currently amended) A method of silencing expression of a target gene, comprising:  
~~introducing into a host cell, an hsiRNA mixture a plurality of fragments made according to claim 1 or claim 11,~~  
wherein the nucleotide sequence for each ~~siRNA fragment~~  
~~in the mixture~~ has a sequence that is complementary to the target gene.
13. (currently amended) A purified set of double-stranded RNA fragments, comprising a plurality of overlapping fragments of a size in the range of 1~~about~~ about 15-30 nucleotides, the fragments in the set collectively representing a substantial portion of a sequence of one or more large double-stranded RNAs from which the

fragments are derived by ~~in-vitro~~ *in vitro* cleavage with a purified enzyme, one strand of each of the large double-stranded RNA having a sequence complementary to part or all of a target messenger RNA.

14. (original) A set of fragments according to claim 13, wherein the substantial portion is greater than about 50% of the sequence of the large double-stranded RNA.

15. (original) A set of fragments according to claim 13, wherein the substantial portion is greater than about 65% of the sequence of the large double-stranded RNA.

16. (original) A set of fragments according to claim 13, wherein more than about 30% of the RNA fragments have a fragment size of about 18-25 base pairs.

17. (original) A set of fragments according to claim 13, wherein at least one fragment and as many as 100% of fragments in the set are capable of causing cleaving the target mRNA in a cell.

18. (original) A set of fragments according to claim 17, wherein at least about 50% of the fragments are capable of causing cleavage of the mRNA.

19. (original) A set of fragments according to claim 17, wherein at least about 75% of the fragments are capable of causing cleavage of the mRNA.

20. (original) A set of fragments according to claim 13, capable of RNA silencing in vivo when introduced into a eukaryotic cell.
21. (withdrawn) A method of creating a library of DNA clones from an hsiRNA mixture, each clone expressing one or more double-stranded RNA fragments from the hsiRNA mixture, the method comprising:
  - (a) denaturing the hsiRNA mixture to form a mixture of unpaired RNA strands;
  - (b) ligating to a 3' end of the unpaired RNA strands, a first single-stranded DNA primer and to a 5' end of the unpaired RNA strand, a second single-stranded DNA primer;
  - (c) reverse transcribing the chimeric DNA-RNA products of step (b) to form complementary DNA fragments; and
  - (d) inserting one or more DNA fragments into a vector to form the library of clones.
22. (withdrawn) A method according to claim 21, wherein step (c) further comprises performing a polymerase dependent amplification of the DNA fragments.
23. (withdrawn) A method according to claim 21, wherein the 5' end of the RNA strand in step (b) is dephosphorylated.
24. (withdrawn) A method according to claim 23, wherein the 3' end of the RNA strand in step (b) is a 3' hydroxyl

end and where the first DNA primer has both a 5' and a 3' phosphate, the first primer being ligated to the 3' end prior to the second primer.

25. (withdrawn) A method according to claim 24, wherein the RNA strand ligated to the first primer of step (b) is phosphorylated and ligated to the second primer, wherein the second primer is non phosphorylated on the 3' ends.

26. (withdrawn) A method of creating a library of clones, each clone corresponding to one or more double-stranded RNA fragments from an hsiRNA mixture, the method comprising:

- (a) denaturing the hsiRNA mixture to form a mixture of unpaired RNA strands;
- (b) enzymatically removing the 5' phosphate from each strand in the mixture;
- (c) ligating to the 3' hydroxyl end of each strand a DNA primer having both a 5' and a 3' phosphate;
- (d) enzymatically phosphorylating the 5' end of the resulting species;
- (e) ligating to the 5' phosphorylated end of each strand, a second DNA primer having nonphosphorylated 3' termini;
- (f) reverse transcribing the chimeric DNA-RNA products of step (e) to form complementary DNA fragments; and
- (g) inserting one or more DNA fragments into a vector to form the library of sequences.

27. (withdrawn) A method according to claim 26, wherein step (f) further comprises performing polymerase-dependent amplification of the DNA fragments.

28. (withdrawn) A method according to claim 26, wherein the vector is pUC19 or a Litmus vector.

29. (withdrawn) A kit for preparing an hsiRNA mixture, comprising: an preparation of RNaseIII, and an RNase buffer containing manganese ions in the range of about 5 mM-100 mM and optionally reagents for synthesizing a large double-stranded RNA.

30. (withdrawn) A method of obtaining a large double-stranded RNA molecule, comprising;

- (a) inserting a DNA fragment or library of DNA fragments encoding a double-stranded RNA into a vector having cloning sites flanked by opposing T7 promoters;
- (b) performing in vitro or in vivo transcription; and
- (c) obtaining the large double-stranded RNA molecule.

31. (withdrawn) A method of reducing expression of one or more target genes in a eukaryotic cell, comprising:

- (a) introducing into the cell, a set of hsiRNA fragments according to claim 13, wherein the large dsRNA is complementary to all or part of a messenger RNA transcript of each of the target genes; and

(b) reducing the expression of the one or more target genes in the eukaryotic cell compared to expression of the genes in the eukaryotic cell absent the hsiRNA.

32. (withdrawn) A method of reducing expression of one or more target genes in a eukaryotic cell, comprising:

introducing into the cell, one or more DNA clones made according to claim 21 or 26, wherein the DNA clones express siRNA fragments suitable for reducing expression of the target eukaryotic cell compared to expression of the genes in the eukaryotic cell absent the DNA sequences.

33. (withdrawn) A method of claim 31 or 32, wherein the eukaryotic cell is present in a mammal such that reducing expression of the one or more target genes cause a phenotypic change.

34. (withdrawn) A method of claim 33, wherein the phenotypic change provides a treatment for a disease in the mammal.

35. (withdrawn) A method according to claim 34, wherein the phenotypic change is an enhancement of a desired characteristic in the mammal.

36. (withdrawn) A method according to claim 33, wherein the phenotypic change is diagnostic for a selected phenotype.

37. (withdrawn) A method according to claim 31 or 32, wherein the reduced expression of a gene is a tool for analyzing a biochemical pathway in which the gene product functions.
38. (withdrawn) A method according to claim 37, wherein the biochemical pathway may be further analyzed in combination with a diagnostic reagent.
39. (withdrawn) A method according to claim 38, wherein the diagnostic reagent is one or more antibodies.
40. (withdrawn) A method according to 31 or 32, wherein the eukaryotic cell is present in a non-human animal.
41. (withdrawn) A method according to claim 31 or 32, wherein the eukaryotic cell is a component of a transgenic animal is created from a fertilized oocyte containing the DNA sequence.
42. (withdrawn) A rapid discovery method for identifying an hsiRNA mixture which is capable of increased gene silencing of a target gene, comprising:
  - (a) synthesizing a plurality of large dsRNAs each large dsRNA having a sequence complementary to a segment of a target gene;
  - (b) digesting each of the large dsRNA with RNaseIII in the presence of a manganese ions to produce a corresponding hsiRNA mixture;

- (c) introducing each hsiRNA mixture into a eukaryotic cell to determine whether gene silencing occurs; and
- (d) determining which of the hsiRNA mixtures caused increased gene silencing.

43. (withdrawn) A method according to claim 42, wherein step (d) further comprises combining a first hsiRNA mixture with a second hsiRNA mixture for increasing gene silencing.

44. (withdrawn) A method according to claim 42, further comprising:  
selecting individual siRNA fragments from hsiRNA mixtures and introducing the individual siRNA fragments into a eukaryotic cell to achieve desired gene silencing

45. (withdrawn) A method of identifying a sequence corresponding to an siRNA from a cleavage site in a mRNA, comprising:

- (a) obtaining an hsiRNA mixture enzymatically;
- (b) introducing the hsiRNA into a cell;
- (c) extracting cleaved mRNA from the cell;
- (d) determining the sequence of terminal nucleotides at the cleavage site of the siRNA cleaved mRNA; and
- (e) identifying the siRNA sequence from the cleavage site sequence and neighboring nucleotides from the intact mRNA.

46. (withdrawn) A method according to claim 45, wherein the step of determining the sequence further comprises using labeled extension DNA primers.
47. (currently amended) A purified set of double-stranded RNA siRNA fragments according to claim 13, wherein the fragments bind comprising double stranded RNA of about 15-30 nucleotides that bind specifically to mRNA to initiate cleavage of the mRNA.